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14. ABSTRACT

Small mimics of host defense proteins (smHDPs) were screened for anti-film activity. The most potent compounds eradicated MRSA biofilms at concentrations superior to gentamycin and other commonly used antibiotics. Select compounds also demonstrated activity against E. coli and P. aeruginosa biofilm cultures. Several compounds were also tested in an alternate in vitro biofilm assay for evaluation in a topical wound biofilm model. The compounds showed strong in vitro activity against S. aureus and P. aeruginosa biofilms and activity was superior over

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Richard Scott
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 484-598-2336

Report Title

Biomimetics for Treating Biofilm-Embedded Infections

ABSTRACT

Small mimics of host defense proteins (smHDPs) were screened for anti-film activity. The most potent compounds eradicated MRSA biofilms at concentrations superior to gentamycin and other commonly used antibiotics. Select compounds also demonstrated activity against E. coli and P. aeruginosa biofilm cultures. Several compounds were also tested in an alternate in vitro biofilm assay for evaluation in a topical wound biofilm model. The compounds showed strong in vitro activity against S. aureus and P. aeruginosa biofilms and activity was superior over mupirocin ointment. Seven smHDPs were tested in a stringent mouse biofilm model examining activity against biofilm-impregnated catheters implanted subcutaneously. None of the PMX compounds tested in the model were efficacious. Several compounds were evaluated in a topical wound biofilm model. MRSA biofilms were pre-formed for 24 hours in partial-thickness mouse wounds and treated with hydrogel formulations. Both compounds achieved log10 reductions of ~5-6 with twice daily application and log10 reductions of ~3-4 even when applied only once daily. Although the goal of identifying biofilm-active smHDPs following systemic administrations, we have been successful in identifying highly active smHDPs in a topical wound biofilm model. These results indicate that smHDPs are promising candidates for topical treatment of biofilm infections in wounds.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Small, nonpeptidic mimics of host defense proteins exhibit potent killing activity against Gram-positive and Gram-negative biofilm cultures. 52nd ICAAC - Interscience Conference on Antimicrobial Agents and Chemotherapy; Sep 9–12, 2012; San Francisco, CA

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Paper

TOTAL:

Patents Submitted

Hybrid compounds and methods of making and using the same

Polyclic compounds and methods of making and using the same

Cyclic compounds and methods of making and using the same

Patents Awarded

Awards

None

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Y. Zha	0.08	
A. O. Tezgel	0.25	
FTE Equivalent:	**0.33**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Y. Li	0.13
F. Sgolastra	0.50
K. Zhang	0.63
B. Fu	0.13
FTE Equivalent:	**1.39**
Total Number:	**4**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Y. Zha
A. O. Tezgel
Total Number: 2

Names of other research staff

NAME	PERCENT_SUPPORTED
Richard Scott	0.05
Dahui Liu	0.75
Claudia Budu	0.16
Meagan Corrigan	0.59
FTE Equivalent:	**1.55**
Total Number:	**4**

Sub Contractors (DD882)

1 a. University of Massachusetts - Amherst

1 b. Office of Grant & Contract Administrati
Research Administration Bldg.
Amherst MA 010039342

Sub Contractor Numbers (c): UMass OGCA 104-1079

Patent Clause Number (d-1): FAR 52.227-11

Patent Date (d-2): 12/1/2007 12:00:00AM

Work Description (e): Medicinal chemistry for optimization of smHDP activity against Gram-positive and Gram-negative

Sub Contract Award Date (f-1): 5/2/2011 12:00:00AM

Sub Contract Est Completion Date(f-2): 9/15/2012 12:00:00AM

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1 b. Office of Grants and Contracts
University of Massachusetts Amherst
Amherst MA 01003

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Inventions (DD882)

Scientific Progress

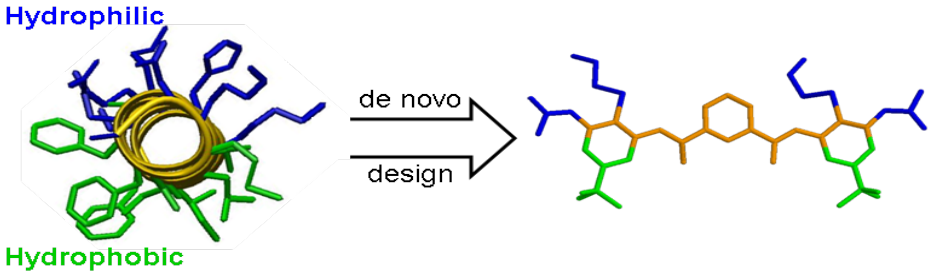
See attachment

Technology Transfer

FINAL REPORT; 09/15/2010 – 09/15/2012
CONTRACT W911NF-10-C-0111
Biomimetics for Treating Biofilm-Embedded Infections

INTRODUCTION

Host defense proteins (HDPs) are an essential component of the innate immune system and display broad-spectrum action against bacteria, yeast, and fungus by specifically disrupting their membranes rather than binding to specific molecular targets. Importantly, this mechanism is associated with a lower risk for the development of resistance. It appears that HDPs are ideal therapeutic agents to treat infections. However, significant problems with stability, tissue penetration and toxicity have hampered their clinical progress. PolyMedix has developed a series of small nonpeptidic mimics of the HDPs (smHDPs) which have robust activity against bacteria and markedly lower toxicity. The approach is to capture the structural and biological properties of the HDPs on a fully synthetic, nonpeptidic framework that would be less expensive to produce, have better tissue distribution, and be much easier to fine-tune structurally to improve activity and minimize toxicity.



Chronic infections, including pulmonary pneumonia, cystic fibrosis, urinary tract infections, osteomyelitis and endocarditis are often associated with microbial biofilm formation. Implanted devices such as prosthetic joints, vascular and urinary catheters, artificial heart valves, pacemakers and cosmetic surgery implants have found increased utility in modern medical practice and are common examples of biofilm-associated infections. Bacteria within biofilms are intrinsically less susceptible to antibiotics because of their altered physiological state under sessile growth conditions and restricted access of the antibiotic throughout the biofilm. As a result, current antibiotics and therapy are either ineffective or less efficacious against biofilm-associated infections as compared to acute infections. We have examined the activity of a set of smHDPs against Gram-positive and Gram-negative biofilm cultures to assess their potential utility as effective antimicrobial agents for treatment of biofilm-associated infections.

SPECIFIC AIMS

The goal for this Phase 2 STTR proposal is to identify smHDPs that are active against pathogens commonly associated with biofilm infections. *In vitro* and proof-of-concept *in vivo* studies are being done to identify lead compounds suitable for further therapeutic development. A medicinal chemistry effort is being continued to optimize activity and safety in the lead series. More specifically:

Aim 1. Optimize smHDP SAR for activity against target pathogens with a focus on *P. aeruginosa* and *A. baumannii*. Efforts will focus on two main targets: 1) optimizing leads for *in vivo* activity against MDR biofilm pathogens like MRSA, *S. aureus*, *S. epidermidis* and other coagulase-negative species as well as *E. faecalis and E. coli*; and 2) improving activity for two other important pathogens *P. aeruginosa* and *A. baumannii*.

Aim 2. Identification of lead compounds active *in vitro* against biofilm cultures of target pathogens. Minimum biofilm eradication concentrations (MBEC) will be defined in a 96 well plate assay. Compounds exhibiting MBICs ≤ 2 ug/ml and MBECs ≤ 50 ug/ml will be evaluated in a long-term colony biofilm model to determine activity over 7 days and potential outgrowth over 14 days. Compounds that are robustly active at concentrations ≤ 10 ug/ml (EC_{50}s) will be evaluated in Aim 3. To prioritize compounds and help interpret *in vivo* efficacy results, *in vitro* assays will be done to measure the level of protein binding, and stability in plasma and liver hepatocytes.

Aim 3. Demonstration of efficacy in an *in vivo* proof-of-concept biofilm infection model. Compounds selected in Aim 2 will be tested for efficacy in treating biofilm infections on implanted catheters. Reductions in bacterial burden in the biofilm during treatment and relapse periods will be examined. Significant improvement in efficacy relative to known antibiotics, without relapse growth, is targeted. Positive results will define the lead series for further testing and chemical optimization, substantiate their pharmaceutical potential and justify the expense in cost and resource support for further development.

RESEARCH RESULTS

Aim 1: Medicinal chemistry. The goal of the medicinal effort was to achieve broad spectrum activity against Gram-positive and Gram-negative bacteria and good selectivity versus mammalian cell types. Three structural series were investigated: arylamide PMX519 analogs, hybrid arylurea/ethers and benzimidazoles. Among all three series, PMX519 series is the most studied. Several arylamides, hybrid arylurea/ethers and benzimidazoles have improved activity/safety profiles relative to PMX519. Eight compounds were efficacious against *S. aureus in vivo*. The activity against Gram-negative organisms was more difficult to achieve compared to Gram-positive organisms. It was found that a strong positive correlate for *in vivo* activity against *E. coli* is activity *in vitro* (MICs ≤ 3 μg/ml) in the presence of serum. Based on the guidance of MIC in the presence 40% mouse serum, benzimidazole PMX1405 was identified and proved to be active against *E.coli in vivo*.

Arylamide 519 series. The arylamide series have been extensively studied around PMX519, a symmetrical pyrimidine diamide. PMX519 is broadly active against the five organisms (three Gram-negative, two Gram-positive) in the primary screen. However, PMX519 showed no efficacy *in vivo*. The lack of efficacy was suspected to be due to several factors: low metabolic stability, lack of activity in the presence of serum and poor solubility *in vivo*. Medicinal chemistry was undertaken to address each of these issues. The chemistry plan consisted of modifications on the sidechain, center ring and the linker between the sidechain and the backbone. After systematic work, it was found that by fine-tuning overall polarity and adding structural contraints with cyclic groups, activity against *S. aureus* can be achieved *in vivo*. It was also found that *in vitro* activity in serum is not a requirement for in vivo activity for *S. aureus*. The lead compounds that were efficacious in a mouse thigh burden model include: PMX1147, PMX1285, PMX1442, PMX1443 and PMX1445. Two of compounds, PMX1091 and PMX1363, shoed partial efficacy. In Table 1, the *in vitro* profile and structure modifications of these compounds are listed.

Table 1. Arylamide lead compounds.

Compound	MIC (μg/ml)							Cytotoxicity (EC_{50}; μM)			Structural modification
	EC	EC +40%ms	SA	SA +40%ms	EF	PA	KP	3T3	HG2	RBCs	
PMX519	1.56	25	1.56	12.5	1.56	6.25	3.13	429.7	>1000	7.8	
PMX1147	6.25	25	1.56	3.13	3.13	6.25	3.13	732.7	525.5	23.91	Extra polar sidechain
PMX1285	3.13	50	1.56	3.13	1.56	6.25	3.13	243.5	415.9	80.2	Cyclic sidechain, ether linker
PMX1442	0.78	>50	0.195	12.5	0.195	1.56	0.78	180.71	601.32	26.0	Cyclic sidechain
PMX1443	0.78	>50	0.39	25	1.56	1.56	0.78	104.43	103.19	3.9	Extra polar sidechain
PMX1445	3.13	50	0.195	12.5	0.78	6.25	3.13	973.19	>1000	27.9	Extra polar sidechain
PMX1091	3.13	>50	1.56	6.25	1.56	6.25	3.13	388.8	723.8	131.0	Guanidino group
PMX1363	1.56	50	0.78	6.25	1.56	3.13	3.13	421.8	262.2	20.4	Cyclic sidechain

ms: mouse serum; ND: Not Determined; EC: E. coli 25922; SA:S. aureus 27660; EF: E. faecalis 29212; PA: P. aeruginosa 10145; KP: K. pneumoniae 13883; 3T3: mouse 3T3 fibroblasts; HG2: human transformed liver HepG2 cells; RBCs: isolated human erythrocytes

Unlike activity against Gram-positive bacteria, it was found that *in vitro* activity in serum was required for *in vivo* efficacy against Gram-negative *E. coli*. Overall polarity seemed to have little effect on Gram-negative serum activity. The activity was more related to the conformation of the backbone. When hydrogen bonding capabilities of the proximal sidechain is removed, serum activity against *E.coli* is improved. Moreover, when the center pyrimidine ring of PMX519 is replaced by pyrazine, the symmetry of the molecule changes from mirror image to c2 symmetry, and the resulting compound gains activity in serum against *E. coli*. However, the gain of *E. coli* serum activity is always paired by the lost of broad spectrum activity. No arylamide is efficacious against *E. coli* in thigh burden study up to date.

Table 2. Modifications that improve serum activity against *e.coli*

Compound	MIC (μg/ml)							Cytotoxicity (EC_{50}; μM)			Structural modification
	EC	EC +40%ms	SA	SA +40%ms	EF	PA	KP	3T3	HG2	RBCs	
PMX519	1.56	25	1.56	12.5	1.56	6.25	3.13	429.7	>1000	7.8	
PMX869	6.25	6.25	0.39	1.56	12.5	6.25	6.25	161.8	193.8	15.2	Removal of H bond
PMX871	0.78	6.25	0.098	0.78	3.13	25	3.13	258.7	910.6	>1000	Removal of H bond
PMX1273	3.13	6.25	0.78	0.78	6.25	>50	12.5	500.0	>1000	>1000	Removal of H bond
PMX1312	3.13	3.13	0.78	0.39	0.78	50	12.5	751.0	392.0	>1000	Removal of H bond
PMX1256	6.25	6.25	3.13	25	6.25	6.25	12.5	189.0	251.8	9.8	C2 symmetry

ms: mouse serum; ND: Not Determined; EC: E. coli 25922; SA:S. aureus 27660; EF: E. faecalis 29212; PA: P. aeruginosa 10145; KP: K. pneumoniae 13883; 3T3: mouse 3T3 fibroblasts; HG2: human transformed liver HepG2 cells; RBCs: isolated human erythrocytes

Arylurea-ether series. In order to expand the chemical space of the scaffold, one strategy is to combine existing active series creating hybrid compounds. Arylurea-ether is the combination of the efficacious arylurea series and compact arylether series. The first hybrid compound PMX842 showed activity that was comparable to the broadly active PMX100 (best arylurea). The MTD is 30 mg/kg, improved form 20 mg/kg of PMX100. However, it is cytotoxic. To improve its selectivity, additional polar groups were substituted on the central ring (PMX1056 and PMx1057). Indeed, the selectivity was greatly improved (Table 3). The aniline moiety of PMX1056 was replaced by a di-thioether aniline in PMX1174 and the activity was retained if not slightly improved. Removal of one thioether sidechain in PMX1175 resulted in the reduction of activity.

Table 3. SAR of hybrid Arylurea-ether

Compound	MIC (μg/ml)							Cytotoxicity (EC_{50}; μM)			Structural modification
	EC	RBCs	SA	SA +40% ms	EF	PA	KP	3T3	HG2	RBCs	
PMX842	1.56	>50	3.13	50.0	1.56	6.35	3.13	52.6	61.8	23.1	
PMX1065	3.13	>50	3.13	12.50	1.56	6.25	3.13	186.1	395.3	130.1	extra polar sidechain
PMX1057	6.25	>50	3.13	12.5	1.56	12.5	6.25	255.5	409.0	247.2	extra polar sidechain, cyclic sidechain
PMX1174	3.13	50	1.56	6.25	0.78	3.13	3.13	142.1	191.0	187.8	extra polar sidechain, less polar linker
PMX1175	6.25	50	1.56	12.5	1.56	3.13	12.5	160.1	238.0	136.9	polar sidechain at different location

ms: mouse serum; ND: Not Determined; EC: E. coli 25922; SA:S. aureus 27660; EF: E. faecalis 29212; PA: P. aeruginosa 10145; KP: K. pneumoniae 13883; 3T3: mouse 3T3 fibroblasts; HG2: human transformed liver HepG2 cells; RBCs: isolated human erythrocytes

Benzimidazole series. When the proximal sulfurs of PMX519 is replaced with nitrogens, the benzimidazole compound PMX1344 is generated by a dehydration reaction. PMX1344 has reasonable broad spectrum antibacterial activity, a MTD value that is greater than 20 mg/kg and accordingly was selected for chemical optimization. Unlike the arylamide PMX519, the side ring of benzimidazole is not rigidified by the hydrogen bond between the proximal liker and the center ring. For this reason, it is observed that activity in the benzimidazole series is quite sensitive to center ring modifications. When the pyrimidine ring is replaced by para-substituted pyrazine, the resulting compound, PMX1405, gained serum activity against *E.coli*. PMX1405 has C2 symmetry, similar to the serum-active arylamide, PMX1256. However, when the center ring is para-substituted benzene, the activity is lost across the panel of bacteria (PMX1448). For pyridine center rings, the meta substituted PMX1546 is inactive, while the para-substituted PMX1431 retained some activity. The conformation of the 2,5 substituted thiophene compound PMX1449 is in-between para and meta substituted benzene, and has moderate activity. PMX1405 is the Gram-negative lead compound from the benzimidazole

series. It is active against *E. coli* in a mouse thigh burden model, which is correlated with its *in vitro* serum activity. PMX1344 has also shown *in vivo* efficacy against *S. aureus* in mouse thigh burden model.

Table 4. SAR of benzimidazoles.

Compound	MIC (µg/ml)							Cytotoxicity (EC_{50}; µM)			Center ring
	EC	EC +40%ms	SA	SA +40% ms	EF	PA	KP	3T3	HG2	RBCs	
PMX1344	6.25	25	1.56	3.13	1.56	3.13	3.13	66.4	87.8	580.8	meta substituted pyrimidine
PMX1405	1.56	0.78	6.25	3.13	6.25	6.25	12.5	1177.1	912.75	48.9	para substituted pyrazine
PMX1431	6.25	>50	0.39	25	6.25	25	25	281.4	545.24	>1000	para substituted pyridine
PMX1448	25	>50	0.78	25	25	>50	50	579.93	627.53	>1000	para substituted benzene
PMX1449	6.25	50	0.39	25	6.25	25	12.5	229.33	254.9	273.1	2, 5 substituted thiophene
PMX1546	>50	>50	12.5	>50	6.25	50	>50	210.93	222.01	>1000	meta substituted pyridine

ms: mouse serum; ND: Not Determined; EC: E. coli 25922; SA:S. aureus 27660; EF: E. faecalis 29212; PA: P. aeruginosa 10145; KP: K. pneumoniae 13883; 3T3: mouse 3T3 fibroblasts; HG2: human transformed liver HepG2 cells; RBCs: isolated human erythrocytes

Aim 2; Biofilm activity. Target pathogens frequently associated with biofilm infections include *S. aureus*, MRSA, *E. coli, P. aeruginosa,* and *A. baumannii*, among others. The standard assay for testing the antibiotic susceptibility of bacteria is minimum inhibitory concentration (MIC), which tests the sensitivity of the bacteria in their planktonic phase. There are several assays which measure anti-biofilm activity. We elected to use a stringent endpoint of total biofilm eradication to measure the activity of compounds since biofilms are commonly associated with re-occurrence of bacterial infection and their eradication, rather than mere inhibition of growth, would be expected to minimize frequencies of infection re-occurrence. Initially, biofilms were established on a 96 well peg devise (Innovotec, MBEC Biofilm Technologies) where bacterial cultures are incubated for 2 days for determination of minimum biofilm eliminating concentrations (MBEC); the lowest concentration of compound where no viable bacteria are recovered from the biofilm culture. Replication of results obtained with this method indicated that there was some variability in the MBEC values with several compounds. Therefore, a second assay was established to determine if more reproducible results could be obtained. With the alternate method, biofilm cultures are established for 2 days on filter disks in 24 well plates and treated with compound for 24 hours. Viability is measured following disruption of the biofilm by sonication, serial dilution and plating for determination of cfus/ml. This method provides an alternate substrate for biofilm formation and appears to provide more reproducible quantification of biofilm growth due to more uniform sonication conditions than provided by the 96 well peg device. From a screen of 160 smHDPs selected from the PMX compound library based on bacterial susceptibility (MIC) and low cytotoxicity, 82 compounds had MBEC values ≤ 64 µg/ml (Table 1). Dose-response studies have been conducted with 41 of these compounds. The most potent compounds had MBEC values of 16 µg/ml and 24 compounds had anti-MRSA biofilm activity (MBECS = 16 - 32 µg/ml), superior to gentamycin and other commonly used antibiotics reported to have biofilm activity.

Table 1. Anti-MRSA biofilm activity of smHDPs with low cytotoxicity

smHDP	Activity (µg/ml) vs. MRSA 33591		Cytotoxicity (µM)	
	MIC	MBEC	3T3	HepG2
648	2	**16**	113.6	120.5
675	0.25	**16**	79.9	188.2
677	1	**16**	306.8	341.0
741	1	**16**	270.8	604.9
817	0.5	**16**	866.3	>1000
1102	1	**16**	256.9	386.4

1257	1	**16**	683.6	710.5
1284	0.5	**16**	74.4	119.1
1388	1	**16**	161.9	181.6
665	0.5	**16-32**	385.6	>1000
742	1	**16-32**	116.4	0
1273	1	**16-32**	500.0	>1000
1419	0.5	**16-32**	96.95	173.34
1555	0.5	**16-32**	102.05	390.5
100	0.5	**32**	128.0	145.0
229	0.5	**32**	727.0	684.0
231	0.5	**32**	159.0	411.0
255	1	**32**	288.0	459.0
652	2	**32**	189.1	478.4
842	0.5	**32**	52.6	61.8
1050	0.25	**32**	146.3	302.1
1099	0.5	**32**	57.1	105.6
1174	0.5	**32**	142.1	191.0
1420	2	**32**	>1000	>1000
1441	1	**32**	457.1	792.51
243	0.5	**32-64**	308.0	650.0
519	0.25	**32-64**	429.7	>1000
603	0.5	**64**	159.0	99.0
843	0.5	**64**	78.7	131.2
985	0.5	**64**	276.9	345.3
1106	1	**64**	276.9	345.3
1008	0.5	**64**	82.8	151.0
1251	2	**64**	587.0	700.3
1252	1	**64**	36.7	167.3
1263	0.5	**64**	82.4	143.3
1312	0.5	**64**	751.0	392.0
1344	1	**64**	66.4	87.8
1424	8	**64**	>1000	>1000
1459	1	**64**	234.68	396.8
1568	1	**64**	531.9	523.39
rifampicin	0.01	**>128**	NT	NT
tigecycline	1	**>128**	NT	NT
daptomycin	1	**128**	NT	NT
gentamycin	1	**64**	NT	NT

MIC: minimum inhibitory concentration; MIC determinations were made under CLSI conditions.; MBEC: minimum biofilm eradication concentration; MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 33591, 3T3: mouse fibroblasts; HepG2: human liver cells.; Cytotoxicity was determined using CellTiter® Aqueous One Solution Cell Proliferation Assay (Promega); NT: Not Tested

Dose response curves of anti-MRSA biofilm activities for selected compounds from Table 1 measuring viable cfus/ml are shown in Figures 1A. The curves are steep for many of the compounds where complete killing was observed between 1 and 2 doubling dilutions. Similar profiles were seen for 2 of the comparator antibiotics

(Figure 1B), gentamycin and daptomycin that have MBECs of 64 and 128 µg/ml, respectively. Rifampicin had no consistent cidal activity against the MRSA biofilms and tigecycline showed only a 1.5 to 2.0 log10 reduction in viable cfus at the highest concentration tested (128 µg/ml).

Figure 1A. Dose-response profiles for select MRSA biofilm-active compounds.

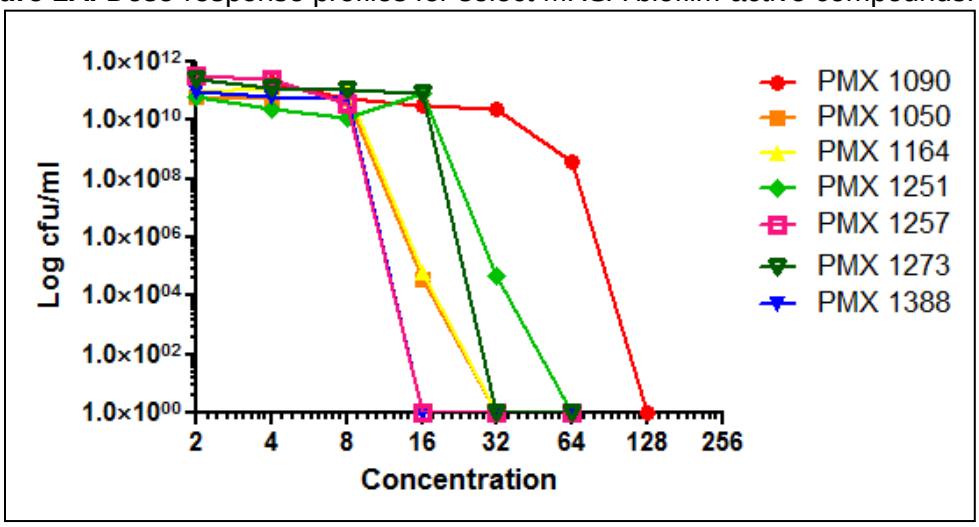
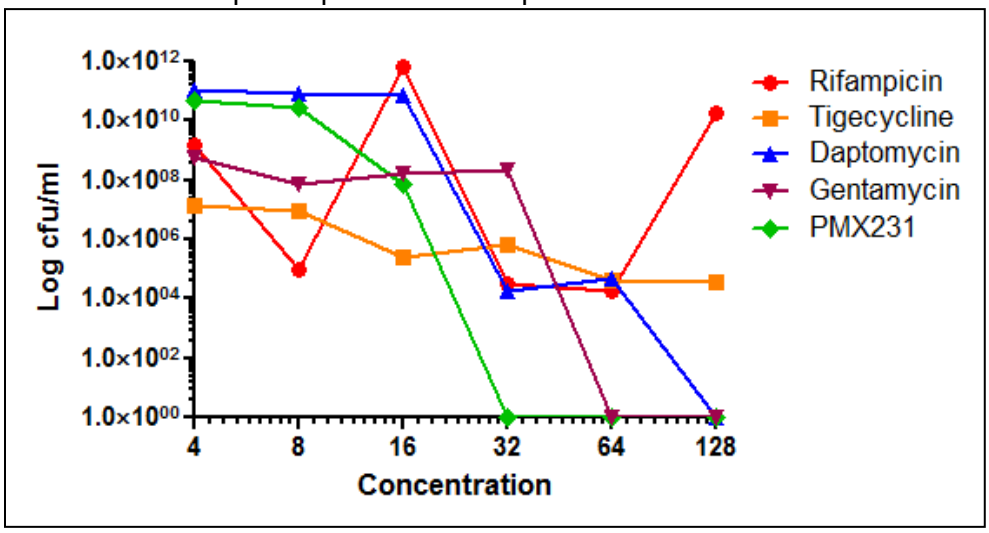


Figure 1B. Dose-response profiles for comparator antibiotics vs. MRSA biofilms



Select compounds showing anti-MRSA biofilm were tested for activity against *E. coli* and *P. aeruginosa* biofilm cultures (Table 2). Most compounds were found to have MBECs in the 32 – 64 ug/ml range. Interestingly, for many of the compounds, activity vs. the Gram-negative biofilm cultures was similar to activity vs. MRSA biofilms, despite greater differences in susceptibility of the planktonic cultures (MICs) (i.e. PMX243, PMX842, PMX1174). This indicates the compounds may have better penetrance in the *E. coli* and/or *P. aeruginosa* biofilms.

Table 2. Anti-Gram negative biofilm activity of smHDPs

smHDP	MIC (µg/ml)			MBEC (µg/ml)		
	MRSA 33591	*E. coli* 25922	*P. aeruginosa* 27853	MRSA 33591	*E. coli* 25922	*P. aeruginosa* 27853
231	0.5	4	8	32	64	128
243	0.5	2	4	32-64	64	64
519	0.25	1	2	32-64	128	64
741	1	4	8	16	32	64
842	0.5	1	4	32	32-64	64

1099	0.5	2	2	32	32	128
1174	0.5	4	4	32	64	64
1284	0.5	8	16	16	32	64-128
1344	1	4	4	64	64	64
1441	1	8	4	32	64	64
MIC: minimum inhibitory concentration; MBEC: minimum biofilm eradication concentration						

Additional compounds were also identified that demonstrated activity against biofilm cultures of *E. coli* but had poor activity against *P. aeruginosa* biofilms (Table 3). These are 2nd priority compounds that will be investigated further only when work with the higher priority compounds in Table 2 is completed.

Table 3. smHDPs with anti-MRSA and *E. coli* biofilm activities but lacking anti-*P. aeruginosa* biofilm activity

smHDP	MIC (µg/ml)			MBEC (µg/ml)		
	MRSA 33591	*E. coli* 25922	*P. aeruginosa* 27853	MRSA 33591	*E. coli* 25922	*P. aeruginosa* 27853
603	0.5	2	4	64	64	256
652	2	16	8	32	64	>256
675	0.25	2	2	16	32	256
1252	1	2	2	64	32	256
1257	1	4	16	16	32	256
1263	0.5	2	2	64	32	>256
1273	1	8	16	32	64	>256
1312	0.5	2	16	64	32	256
1420	2	16	8	32	64	256
MIC: minimum inhibitory concentration; MBEC: minimum biofilm eradication concentration						

From the priority compounds described in Tables 1 and 2, selections of biofilm-active compounds were made for animal studies based on activities against MRSA and Gram-negative biofilms, low cytotoxicity vs. mammalian cells, *in vivo* tolerability in acute toxicity studies (MTDs; Maximum Tolerated Dose) and structural diversity (Table 4). Also, since the model incorporates a 7 day dosing regimen, a repeat dose MTD study was conducted in Balbc mice, the same mouse strain as used in the efficacy model (Table 4), to help design dosing regimens.

Table 4. Lead anti-biofilm compounds for animal testing

smHDP	Series	MBEC (µg/ml) MRSA 33591	Cytotoxicity (µM)		Single dose MTD (IV, mg/kg)	Repeat dose MTD (IP, mg/kg)
			3T3	HepG2		
231	**Arylamide I**	32	159	411	17	17.3
243	**Arylamide I**	32-64	308	650	17	17.2
741	**Arylamide I**	16	271	605	19	19
742	**Arylamide I**	16-32	116	>1,000	20	30
842	**Arylurea**	32	53	62	19	9
985	**Arylamide II**	64	277	345	25	10
1147	**Arylurea**	32	142	191	24	10
1284	**Triaryl**	16	74	119	7	5
1344	**Benzimidazole**	64	66	88	25	10
MBEC: minimum biofilm eradication concentration; MTD: single dose IV, CD-1 mice, free base weight. Cytotoxicity was determined using CellTiter® Aqueous One Solution Cell Proliferation Assay (Promega) in mouse 3T3 fibroblast and human HepG2 liver cells. Single dose MTD: CD-1 mice. Repeat dose MTD: intraperitoneal (IP) QD for 5 days in Balbc mice.						

Plasma stability studies have been completed for seven compounds being evaluated in the mouse biofilm model. All 7 compounds were highly stable in mouse plasma with no loss of parent compound evident over a 2 hour incubation at 37°C (Table 5).

Table 5: Stability of PMX lead compounds in mouse plasma

	% Peak Area Relative to 0 min Timepoint			
smHDP	**0 min**	**30 min**	**60 min**	**120 min**
PMX231	100.0%	118.0%	111.6%	104.9%
PMX243	100.0%	114.3%	121.9%	119.0%
PMX1344	100.0%	121.2%	132.6%	137.8%
PMX842	100.0%	100.2%	92.2%	101.1%
PMX985	100.0%	115.2%	100.6%	112.7%
PMX1147	100.0%	134.4%	135.3%	131.3%
PMX1284	100.0%	121.3%	113.3%	110.4%

Prior to animal testing, activity, including both MICs and MBECs, against the infectious pathogen used in the model, *S. aureus* ATCC 6538, was confirmed for all selected compounds (not shown).

An *in vivo* efficacy model with 7 smHDPs and an antibiotic control (rifampin) was performed in collaboration with the University of North Texas Health Science Center (UNTHSC). The testing was completed in 2 sequential studies; Study A and Study B (Table 6). Briefly, segments of Teflon catheters were infected with *S. aureus* (ATCC 6538) for 2 hours to establish a biofilm on the catheter surface and lumen. The infected catheters were surgically implanted into a subcutaneous pocket and the wound was closed. Beginning 7 days after infection, mice were treated at the indicated IP dosages for 7 days. The catheters were removed 18 hours after the last dose, the biofilms were dispersed by sonication, and viable CFUs were determined by serial dilution and plating. Unfortunately, none of the PMX compounds tested in the model were efficacious.

Table 6: Results of mouse subcutaneous catheter biofilm model

Compound	**Dose (IP; mg/kg)**	**Regimen**	**Mean log_{10} CFU/catheter**	**Standard Deviation**	**Mean Log $_{10}$ Reduction**	
					7 Days	**14 Days**
Study A						
PMX231	20	QD	7.34	0.21	-0.87	-0.23
	10	BID	7.10	0.35	-0.73	-0.08
PMX243	20	QD	7.11	0.18	-0.64	0.00
	10	BID	7.19	0.13	-0.73	-0.08
PMX842	20	QD	7.12	0.33	-0.65	-0.01
	10	BID	7.03	0.15	-0.56	0.08
Rifampin	25	BID	5.21	0.84	1.26	1.90
Control	Day 7	na	6.47	0.60		0.64
	Day 14		7.11	0.35	-0.64	
Study B						
PMX985	10	QD	7.12	0.17	-0.39	0.22
	5	BID	7.26	0.24	-0.52	0.09
PMX1147	10	QD	7.16	0.15	-0.42	0.18
	5	BID	7.17	0.23	-0.43	0.17
PMX1284	5	QD	7.28	0.15	-0.54	0.06
	3	BID	7.13	0.22	-0.40	0.21
PMX1344	8.3	BID	7.21	0.17	-0.47	0.13
	5.5	TID	7.44	0.12	-0.70	-0.09
Rifampin	25	BID	5.32	0.89	1.42	2.03
Control	Day 14	na	7.34	0.16	-0.61	
	Day 7		6.74	0.49		0.61

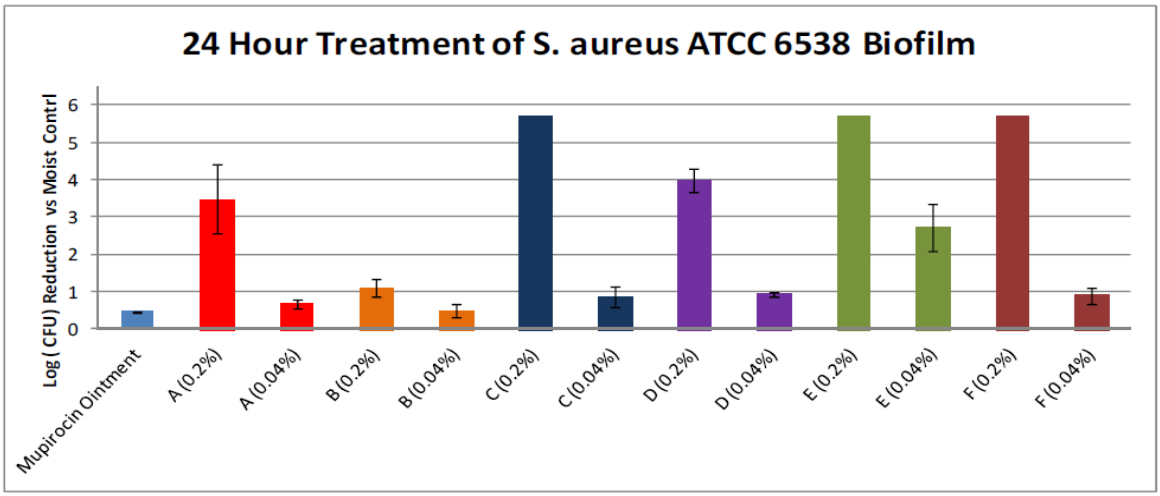
Several compounds have been tested in an alternate *in vitro* biofilm assay for the purpose of selecting leads for evaluation in a topical wound biofilm model. Individual biofilm assemblies consisted of a 13 mm

polycarbonate filter membrane (0.2 µm) topped with a 4 mm collagen disk (Promogran). These assemblies were placed on Tryptic soy agar with 5% sheep's blood and inoculated with 2 or 3 µl of bacterial suspensions of *S. aureus* ATCC 6538 (1x108 CFU/mL) or *P. aeruginosa* ATCC 27312 (2x 108 CFU/mL), respectively. Inoculated assemblies were incubated at 37 °C for 24 hours prior to treatment. For treatments in solution, 150 µl was applied to 10x10 mm Telfa gauze that was then transferred to individual biofilm assemblies. For treatments in formulation, 150 µl was dispensed from syringe onto moistened (50 µl PBS) Telfa gauze and transferred treatment side down to biofilm assemblies. Following 24 hours of incubation at 37 °C, individual treated assemblies were removed, transferred to PBS, thoroughly vortexed and serial 1/10 dilutions were performed in duplicate. For quantification of viable bacteria, 10 µl was spotted on charcoal agar for each dilution series, incubated 18 hours, and dilutions resulting in 10 – 50 colony forming units were counted. Experiments with water solubilized compounds were performed in duplicate and formulated compounds were examined in triplicate.

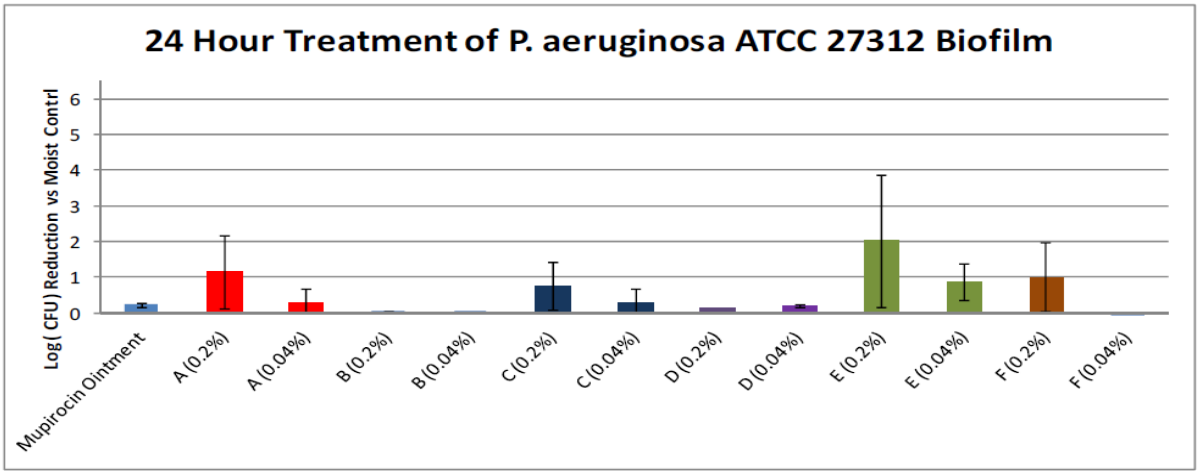
The compounds which were selected for testing are structurally diverse and demonstrated robust anti-biofilm activity in the filter disk assay (Table 1). The compounds are PMX229 (A), PMX648 (B), PMX1174 (C), PMX1257 (D), PMX1284 (E) and PMX1555 (F). For the initial round of in vitro biofilm testing, PMX compounds A-F were dissolved in water at two concentrations (0.04% and 0.2%) and applied to nonstick gauze which was placed in direct contact with *S. aureus* (Figure 2A) or *P. aeruginosa* (Figure 2B) colony biofilms. Quantitative microbiology as described above was performed after 24 hours treatment. Five of the six smHDPs (A, C-F) exhibited strong kill (>3 log reduction) for *S. aureus* biofilms at the 0.2% concentration. Efficacy for *P. aeruginosa* was limited at this concentration but many of the compounds exhibited more robust activity than the positive control comparator, mupirocin ointment (2% w/w of active agent). Based on the dose response seen in Figure 1B, higher concentrations of compound may be more efficacious against the *P. aeruginosa* biofilms (see below).

Figure 2. In vitro activity of solubilized smHDPs against MRSA and P. aeruginosa biofilms

A



B



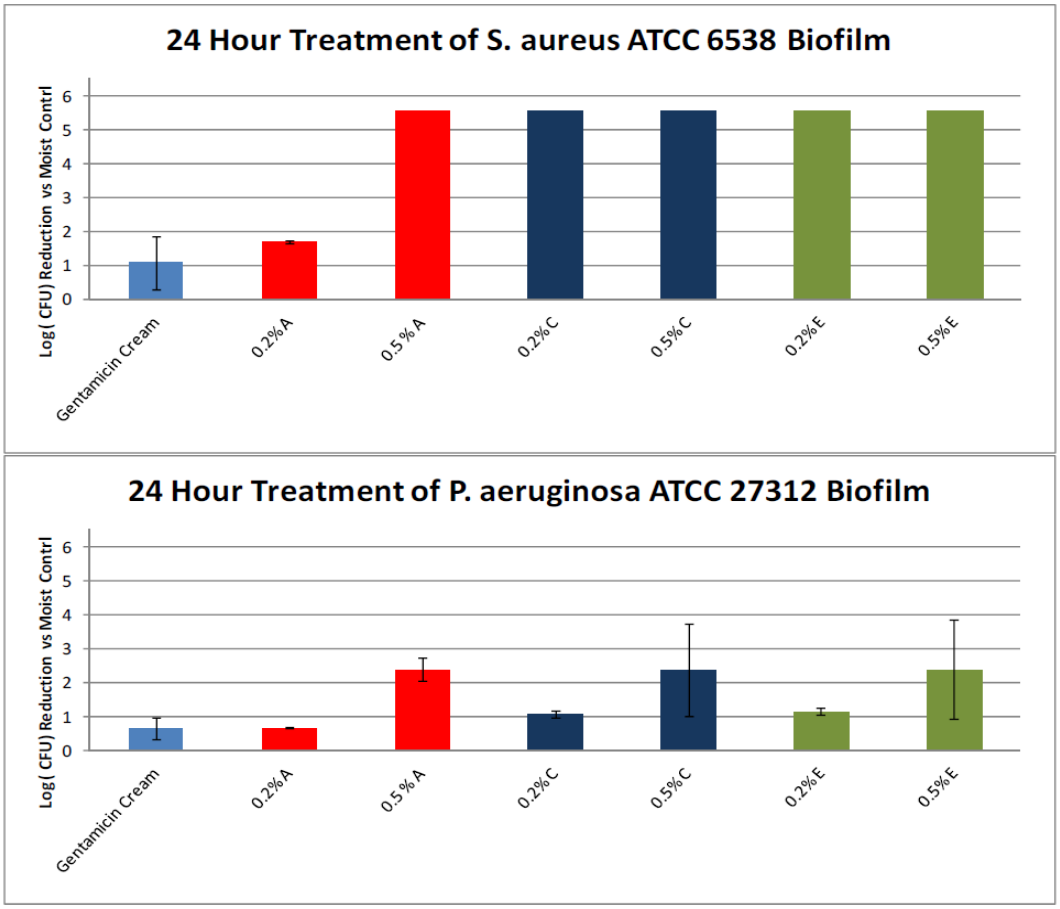
PMX229 (A), PMX1174 (C) and PMX1284 (E) were selected for solvent optimization and animal testing. PMX229 was resuspended in four hydrogel bases, the anticipated formulation for topical use, and tested for activity in a modifiled zone of inhibition assay (Table 7). These experiments were performed in accordance with standard methods for disc assay, except that 6 mm punch holes were made in agar plates and the resulting wells were filled with 130 µl of hydrogels. Based on the results, the HPC base was chosen for further study, and compounds A, C, and E were formulated in this base at 0.2% and 0.5% concentrations.

Table 7. Zones of inhibition for hydrogel formulations of Compound A (PMX229)

	Zone of Inhibition (mm)		Comments
	S. aureus	P. aeruginosa	
3.14% HPC PLACEBO	None	None	
2% HPMC PLACEBO	None	None	
2.8% HEC PLACEBO	None	None	
3.14%HPC 0.2% PMX-A	22	19	
2% HPMC 0.2% PMX-A	22	19	
2.8%HEC 0.2% PMX-A	21	19	Swelled out of the test well
2.4% POL 0.2% PMX-A	21	16	Diffused, no gel left in well

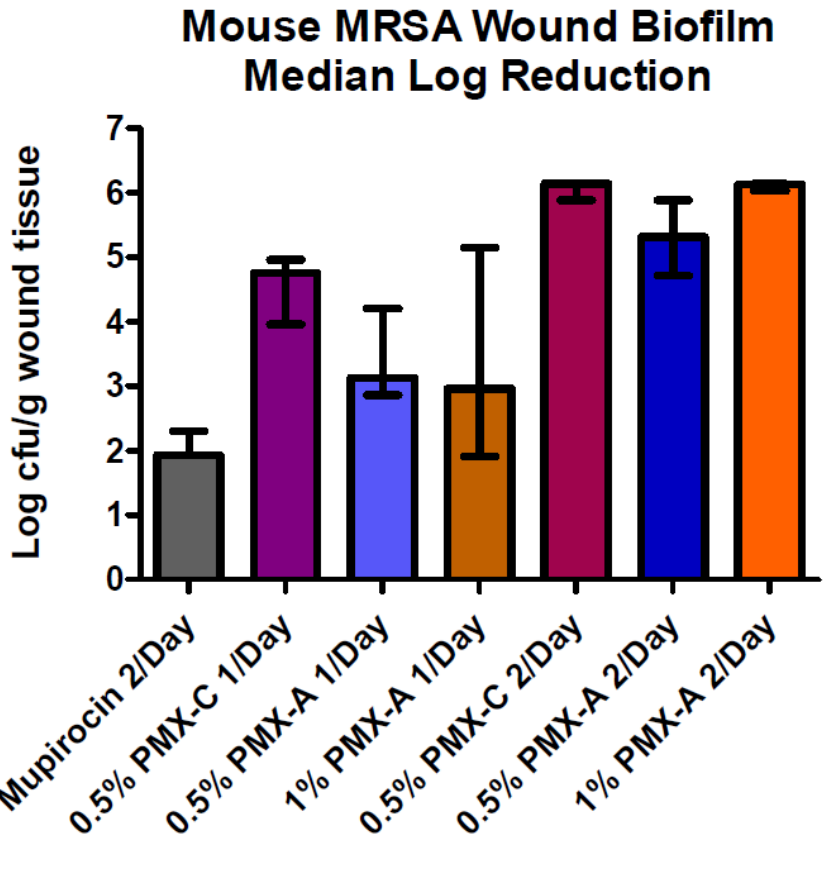
HPC hydrogel formulations containing 0.2% and 0.5% of PMX229 (A), PMX1174 (C) and PMX1284 (E) were tested against *S. aureus* and *P. aeruginosa* colony biofilms (Figure 3). Regarding *S. aureus* biofilm treatment, PMX229 was highly effective (>5 log kill) at the 0.5% dose and PMX-C and E were highly effective (>5 log kill) at both doses. All three compounds also exhibited substantial efficacy at the 0.5% concentration against *P. aeruginosa* (>2 log kill). Based on these *in vitro* results and the more favorable cytotoxicity profiles PMX229 and PMX1174 over PMX1284, PMX224 (A) and PMX1174 (C) were advanced into the animal model and formulated at 0.5% (A and C) or 1% (A only) in the HPC hydrogel base.

Figure 3. *In vitro* biofilm efficacy of smHDPs in HPC Hydgrogel



Partial-thickness mouse wounds in which MRSA biofilms were pre-formed for a 24 hours were treated once or twice daily for two days with HPC formulations of PMX229 (C) or PMX1174 (C) or the positive control comparator, mupirocin ointment. Quantitative microbiology was performed at study end to determine viable MRSA counts (Figure 4). Unfortunately, the mupirocin control group lost samples and is not included in the final analysis due to the small sample size and the high variance of the few remaining data points. Mupirocin control results from a previous recent prior study using this model have been included as a more useful reference point in Figure 4. The median log reductions in viable biofilm bacteria measured were substantial. Both PMX229 and PMX1174 achieved log10 reductions of ~5-6 with twice daily application and log10 reductions of ~3-4 even when applied only once daily. These results indicate that PMX229 and PMX1174, and perhaps other smHDPs, are promising candidates for topical treatment of biofilm infections in wounds.

Figure 4. Activity of smHDPs in a mouse wound biofilm infection model



SUMMARY OF ACCOMPLISHMENTS

Aim 1. Optimize smHDP SAR for activity against target pathogens.

- The goal of the medicinal effort was to achieve broad spectrum activity against Gram-positive and Gram-negative bacteria and good selectivity versus mammalian cell types.
- Three structural series were investigated: arylamide PMX519 analogs, hybrid arylurea/ethers and benzimidazoles. Among all three series, PMX519 series is the most studied.
- Several arylamides, hybrid arylurea/ethers and benzimidazoles have improved activity/safety profiles relative to PMX519. Eight compounds were efficacious against *S. aureus in vivo*.
- The activity against Gram-negative organisms was more difficult to achieve compared to Gram-positive organisms. It was found that a strong positive correlate for *in vivo* activity against *E. coli* is activity *in vitro* (MICs ≤ 3 μg/ml) in the presence of serum.
- Based on the guidance of MIC in the presence 40% mouse serum, benzimidazole PMX1405 was identified and proved to be active against *E.coli in vivo*.

Aim 2. Identification of lead compounds active in vitro against biofilm cultures of target pathogens.

- A screen of 160 smHDPs selected from the PMX compound library based on bacterial susceptibility and low cytotoxicity identified 82 compounds with MBEC values ≤ 64 μg/ml.
- The most potent compounds had MBECs of 16 μg/ml and 24 compounds had anti-MRSA biofilm activities of 16 to 32 μg/ml, superior to gentamycin and other commonly used antibiotics reported to have anti-biofilm activity.
- Select compounds showing anti-MRSA biofilm also demonstrated activity against *E. coli* and *P. aeruginosa* biofilm cultures with MBECs in the 32 – 64 μg/ml range. Interestingly, for many of the compounds, activity vs. the Gram-negative biofilm cultures was similar to activity vs. MRSA biofilms, despite greater differences in susceptibility of the planktonic cultures (MICs). This indicates the compounds may have better penetrance in the *E. coli* and/or *P. aeruginosa* biofilms.
- Additional compounds were also identified that demonstrated activity against biofilm cultures of *E. coli* but had poor activity against *P. aeruginosa* biofilms.
- Selections of biofilm-active compounds were made for animal studies based on activities against MRSA and Gram-negative biofilms, low cytotoxicity vs. mammalian cells, *in vivo* tolerability in single and repeat dose toxicity studies, and structural diversity.
- All compound showed excellent stability in plasma.
- Several compounds have been tested in an alternate *in vitro* biofilm assay for the purpose of selecting leads for evaluation in a topical wound biofilm model. Five of the six smHDPs exhibited strong kill (>3 log reduction) for *S. aureus* biofilms at the 0.2% concentration and activity was superior to the positive control comparator, mupirocin ointment containing 2% w/w of active agent.
- Efficacy for *P. aeruginosa* was more limited at 0.2% concentrations but many of the compounds exhibited better activity than the positive control comparator, mupirocin ointment.
- A hydrogel formulation was identified which showed strong activity against *S. aureus* and *P. aeruginosa* biofilms at 0.5% formulations of active agent and activity was superior over mupirocin ointment.

Aim 3. Demonstration of efficacy in an in vivo proof-of-concept biofilm infection model.

- 7 smHDPs and an antibiotic control (rifampin) were tested in a stringent mouse biofilm model examining activity against biofilm-impregnated catheters implanted subcutaneously for 7 days prior to treatment. Unfortunately, none of the PMX compounds tested in the model were efficacious whereas efficacy was apparent with the control comparator.
- Several compounds were evaluated in a topical wound biofilm model. MRSA biofilms were pre-formed for 24 hours in partial-thickness mouse wounds and treated once or twice daily for two days with hydrogel formulations of 2 smHDPs, PMX229 or PMX1174. Both compounds achieved log10 reductions of ~5-6 with twice daily application and log10 reductions of ~3-4 even when applied only once daily. Activity was superior over historical control results with mupirocin ointment.
- Although the goal of identifying biofilm-active smHDPs following systemic administrations, we have been successful in identifying highly active smHDPs in a topical wound biofilm model. These results indicate that PMX229 and PMX1174, and perhaps other smHDPs, are promising candidates for topical treatment of biofilm infections in wounds.